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# Fibrinogen domain of FREP1 is a broad spectrum malaria transmission-blocking vaccine antigen

Guodong Niu[4,5], Caio Franca[1,5], Genwei Zhang[1], Wanlapa Roobsoong[2], Wang Nguitragool[2], Xiaohong Wang[4], Jetsumon Prachumsri[2], Noah S. Butler[3], and Jun Li[1,4*]

[1]Department of Chemistry and Biochemistry, University of Oklahoma, 101 Stephenson Parkway, Norman, Oklahoma 73019, USA

[2]Mahidol Vivax Research Center, Mahidol University Faculty of Tropical Medicine, Bangkok 10400, Thailand

[3] Department of Microbiology and Immunology, University of Iowa, 51 Newton Road, Iowa City, IA 52242, USA

[4] Department of Biological Sciences, Florida International University, Miami, FL 33199, USA

[5] These authors contributed equally to this work

[*] Corresponding author:
Dr. Jun Li
Department of Biological Sciences
Biomolecular Sciences Institute
Florida International University
11200 SW 8th Street, OE 214
Miami, FL 33199
Tel: 305-348-7618
Email: lij@fiu.edu

**Running title**: FBG is a universal malaria TBV antigen

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**Abstract**

FREP1 in mosquito midguts facilitates *P. falciparum* parasite transmission. Fibrinogen-like (FBG) domain of FREP1 is highly conserved (>90% identical) among *Anopheles* species from different continents, suggesting that anti-FBG antibodies may block malaria transmission to all anopheline mosquitoes. Using standard membrane-feeding assays, anti-FREP1 polyclonal antibodies significantly blocked transmission of *P. berghei* and *P. vivax* to *An. gambiae* and *An. dirus* respectively. Furthermore, *in vivo* studies of mice immunized with FBG achieved >75% blocking efficacy of *P. berghei* to *An. gambiae*, without triggering immunopathology. Anti-FBG serum also reduced >81% *P. falciparum* infection to *An. gambiae*. Finally, we showed that FBG interacted with *Plasmodium* gametocytes and ookinetes, revealing the molecular mechanism of its antibody transmission-blocking activity. Collectively, our data support that FREP1-mediated *Plasmodium* transmission to mosquitoes is a conserved pathway, and targeting FBG domain of FREP1 will limit the transmission of multiple *Plasmodium* species to multiple *Anopheles* species.

**Introduction**

Malaria death rates have dropped by 47% between 2000 and 2013 globally, and by 54% in Africa due to applications of several anti-malaria strategies including anti-malaria drugs, insecticide-treated nets, and indoor insecticide spraying. Despite these efforts, more than 587,000 still died and 90% of these deaths occurred in Sub-Saharan Africa in 2014 (1). The rapid spread of drug-resistant malaria parasites and insecticide-resistant mosquitoes along with the absence of efficient vaccines against malaria present major challenges for malaria control. Therefore, new approaches are urgently needed. Transmission blocking vaccines (TBVs) have been considered as a promising measure to combat malaria. TBVs are designed to block parasite development in the mosquito midgut upon ingestion with the human antibodies against antigens from either parasites or mosquitoes.

Human malaria is caused by *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*, of which *P. falciparum* and *P. vivax* are responsible for ~99% of malaria cases. Since only gametocytes can infect mosquitoes, antigens on the surface of gametocytes and/or ookinetes such as Pfs25, Pfs48/45 and Pfs230 have been evaluated as TBV candidates in preclinical studies (2-4). Among them, Pfs25 and its ortholog Pvs25 from *P. vivax* are the only candidates to progress to clinical trials. Pfs25 is a 25-kDa sexual stage specific protein expressed on the surface of the parasite during several sexual developmental stages including gamete, zygote and ookinete (5). Clinical trials of Pfs25 only showed moderate levels of transmission-blocking activity (6), underscoring the need to identify additional and novel antigens for TBV development.

About 30 anopheline mosquito species transmit malaria(7). The major malaria vectors in Africa are *Anopheles gambiae, An. arabiensis* and *An. funestus*. In Asia, the most important species are *An. stephensi* and *An. dirus.* In South America, *An. minimus*, *An. albimanus* and *An. darlingi* are responsible for malaria transmission (2,8,9). To successfully transmit malaria, *Plasmodium* parasites must complete a complex developmental cycle in both human and mosquito hosts. Thus, mosquito midgut molecules that facilitate ookinete invasion are likely to serve as ideal targets for TBVs. Previous studies showed that polyclonal antibodies against mosquito alanyl aminopeptidase 1 (APN1) or carboxypeptidases B (CPB) (2,10) inhibited 73% and 51% parasite development in mosquito midguts respectively using *P. berghei*-mouse infection system.

Since human malaria is caused by several *Plasmodium* species and transmitted by numerous *Anopheles* species, and many endemic areas have both *P. falciparum* and *P. vivax* malaria cases and transmitted by several different *Anopheles* species, an ideal TBV antigen would effectively block malaria transmission of multiple parasite species to multiple mosquito species. We recently reported that FREP1 plays a pivotal role in ookinete invasion of the mosquito midgut (11). FREP1 is a tetramer that localizes within the peritrophic matrix, and facilitates *Plasmodium* invasion through direct binding to gametocytes and ookinetes. In this study, we demonstrate that a highly conserved FBG domain within FREP1 is a broad-spectrum TBV antigen that blocks transmission of multiple *Plasmodium* species to multiple *Anopheles* species, which supports

FREP1-mediated *Plasmodium* invasion to mosquitoes as a conserved pathway. In particular, *in vivo* mouse model demonstrates FBG as a vaccine that blocks >75% transmission of *P. berghei*, better than reported mosquito TBV antigens (APN1, CBP) (2,10). It is worth noting that only three mosquito proteins have been identified suitable for malaria TBV antigens. Membrane feeding assays showed anti-FBG serum blocked >81% transmission of *P. falciparum*, which meets PATH guideline for malaria TBVs for clinical trials (>80%) (12).

## Results

### The FREP1 fibrinogen-like domain is highly conserved in anopheline mosquitoes

We examined the FREP1 orthologs among anopheline mosquitoes to find conserved regions. We obtained 13 orthologs from major malaria vectors in Africa (*An. gambiae*, *An. funestus, An. arabiensis, An. coluzzii*, *An. merus,* and *An. albumnus*), South America (*An. darlingi*), Asia (*An. sinesis, An. stephensi, An. minimus,* and *An. epiroticus*), and Europe (*An. atroparvus*). The results from multi-sequence alignment revealed a highly conserved region between amino acids 463 and 677 of *An. gambiae* FREP1 (Fig. 1a). Detailed analyses of this conserved region found that more than 90% of the protein sequences are identical among all 13 anopheline species (Fig. 1b), suggesting that antibodies raised against this domain might be able to block the transmission of multiple *Plasmodium* species to multiple *Anopheles* mosquitoes. Since this conserved region is a FBG domain, we also compared FREP1 with human fibrinogens α, β, and γ chains. Multiple sequence alignment found less than 10% identical sequences between the mosquito conserved FBG domain and human fibrinogens, supporting that vaccination with recombinant mosquito FREP1 or the FBG domain protein would be unlikely to trigger autoimmune reactions.

### Rabbit Anti-FREP1 antibodies inhibit malaria transmission in *P. berghei* and *P. vivax* in *Anopheles gambiae* and *Anopheles dirus* respectively

Previously, we reported that anti-FREP1 rabbit polyclonal antibodies effectively blocked *P. falciparum* in a major malaria vector, *An. gambiae* (11). Since there is a highly conserved FBG domain among anopheline orthologs, we determined whether anti-FREP1 antibody would also inhibit transmission of other *Plasmodium* species to additional *Anopheles* species. To address this question, *An. gambiae* mosquitoes were fed with *P. berghei* infected blood mixed with rabbit anti-FREP1 serum (1:1 dilution, final titer units: $5x10^4$), and subsequently examined the number of developing oocysts in mosquito midguts. Rabbit pre-immune serum was used as a negative control. The results showed that anti-FREP1 serum significantly reduced the number of *P. berghei* oocysts, compared with the control group that substituted anti-FREP1 serum with pre-immune serum (Fig. 2a). The average number of *P. berghei* oocysts per midgut significantly decreased from 10 in the control group to 3 in the experimental group ($p<0.0001$). The results were consistent in two biological replicates, which were conducted with different *P. berghei*-infected mouse blood.

Next we tested whether anti-FREP1 antibody could inhibit the transmission of another major human malaria pathogen, *P. vivax*, to another major malaria vector in Asia, *An. dirus*. *P. vivax*-infected blood (2 field isolates) was mixed with rabbit anti-FREP1 serum (1:1 dilution, final titer units: $5x10^4$) and fed to *An. dirus*. The results showed that the anti-FREP1 antibody significantly reduced the number of oocysts per midgut more than two folds compared to the control serum (Fig. 2b). Statistical analysis showed that the inhibitory effect of anti-FREP1 antibodies against *P. vivax* infection in *An. dirus* is significant ($p <0.005$). The results were consistent in two biological replicates, which were conducted with different *P. vivax*-infected human blood.

Together, these data support that anti-FREP1 antibodies can block the transmission of multiple species of malaria parasites to multiple mosquito species.

### Experimental immunization of mice with FREP1 does not trigger toxicity or elicit antibodies that cross-react with human fibrinogen

Our sequence alignment displayed a minor degree of homology between mosquito FREP1 and mammalian fibrinogens. Despite this, we also investigated whether immunization of mice with FREP1 causes any autoimmune response. We

expressed the recombinant FREP1 protein in *E. coli* and insect cells and purified the recombinant proteins using Ni-NTA affinity columns (11). For both *E. coli* and insect cell expressed recombinant FREP1, mice were immunized with a series of FREP1 doses (0.2, 2, or 20μg) and boosted with the same dose twice at three-week intervals. Pre-immune human plasma coated ELISA assays were performed to assess the cross reactivity between anti-FREP1 polyclonal antibodies raised in mice and human blood plasma (human fibrinogens). Wells coated with bovine serum albumin or insect cell-expressed recombinant FREP1 were used as negative and positive controls respectively. The results showed no cross-specific recognition between the anti-FREP1 antibodies generated by *E. coli* or insect cell expressed FREP1 to human plasma fibrinogen (Fig. 3a).

Complementarily, we examined whether immunization with recombinant FREP1 triggered toxicity or caused inflammatory immunopathology in mammals. Alanine aminotransferase (ALT) are mainly in liver and liver is very sensitive to inflammation and toxins. Therefore, the activities of ALT in the immunized mice were examined. The positive control was the blood from *P. yoelii*-infected mice, which exhibited high levels of ALT, characteristic of inflammation and liver damage (Fig. 3b). Notably, the levels of ALT activity in the mice injected with *E. coli*- or insect cell-expressed FREP1 protein was similar to the baseline activity observed in naïve mouse serum ($p>0.38$), supporting that immunization of FREP1 does not cause inflammatory autoimmune or toxicity responses.

**Immunizing mice with FBG inhibits the transmission of *P. berghei* to mosquitoes *in vivo* using direct feeding on mice**

Although anti-FREP1 antibodies block the transmission of multiple *Plasmodium* species to multiple *Anopheles* species, only FBG domains are conserved among anopheline mosquitoes. Therefore, we next determined whether the highly conserved FREP1 FBG domain alone was an effective TBV antigen *in vivo* and *in vitro*. We cloned the FREP1 FBG domain (from 463 to 729 aa) and expressed it in *E. coli*. Mice were immunized with purified FBG protein mixed with Alhydrogel adjuvant using an optimal prime-boost regimen. High-titer antibody levels ($3x10^6$ on average) against FBG were achieved in anti-serum. Mice in the control group were immunized with buffer mixed with Alhydrogel under the same regimen. Ten days after second boost, mice were infected with *P. berghei*. Seven days after infection, the parasitemia was about 10% in mice. To induce maturation of gametocytes, the phenylhydrazine hydrochroride in 0.9% NaCl was injected into mice intraperitoneally (60μg/g body weight). Two days late, mice at similar level of infection (parasitemia 10-12%, gametocytemia: 1-1.5%) were used to infect *An. gambiae* directly. Results showed that *An. gambiae* mosquitoes fed on the FBG-immunized mice had 4.2 and 3.5 oocysts per midgut on average in two experiments, which was significantly ($p<0.0004$) fewer than mosquitoes fed on mock-immunized mice that had 16.7 and 19.8 oocysts on average in two experiments (Fig. 4a). Anti-FBG antibodies reduced the number of oocysts by 75% and 82% in these two independent replicates. We collected the mouse sera and measured titer units of anti-FBG antibodies by ELISA endpoint titer assays.

**Anti-FBG anti-serum inhibits the transmission of *P. falciparum* to mosquitoes in membrane-feeding assays**

To test whether mouse anti-FBG serum also exhibits transmission-blocking activity against *P. falciparum*, we added immune serum from immunized mice to *P. falciparum*-infected blood containing gametocytes and fed to mosquitoes using SMFA. The average number of oocyst per midgut was reduced from 17.4 and 7.2 in control groups to 3.3 and 1.2 in experimental groups, respectively, in two replicates (Fig. 4b). The anti-FBG serum significantly ($p<0.0001$) reduced the number of oocysts of *P. falciparum* by more than 81%. These *in vivo* and *in vitro* experiments demonstrate that the conserved FBG alone is a potent universal TBV antigen

**FBG binds sexual stage of ookinetes**

We determined the molecular relationship between FBG and parasites. We previously showed that FREP1 binds to *P. falciparum* (NF54) sexual stage gametocytes and ookinetes (11). In this study, we examined the capacity for the purified FBG domain of FREP1 to bind *P. falciparum* parasites, gametocytes and ookinetes in particular. Non-infected human red blood cells (RBC), *P. falciparum* gametocytes, and ookinetes were fixed on cover slips. After incubation with

insect cell-expressed FBG recombinant protein, we detected parasite bound FBG protein by indirect immunofluorescence assays. The results showed that FREP1 FBG bound gametocytes (Fig. 5, row C), early stage ookinetes (just merged with a sharp tail, Fig. 5, row D) and ookinetes (Fig. 5, row E). The binding signals were not observed when non-infected human RBC were assayed (Fig. 5, row A), confirming that FBG does not bind healthy human RBCs. The red fluorescence intensity for asexual stage parasites was lower (Fig. 5, row B) than gametocytes and ookinetes. Sexual stage parasites and gametocytes are within red blood cells, however, the fluorescence signals depicted gametocytes well (Fig 5, row C). This indicates that FBG binds to parasites directly. Since gametocytes stretch cells, parasites particularly gametocytes inside cells might change red blood cell permeability or make infected cells more fragile to 4% paraformaldehyde treatment than uninfected cells. Additional negative controls included incubation of *P. falciparum* gametocytes with an irrelevant protein (chloramphenicol acetyltransferase, CAT) expressed in the same system. As shown in Fig. 5, row E, there were no binding signals in control groups. Therefore, FBG fragment is a functional domain of FREP1 that is responsible for the interaction between FREP1 protein and *Plasmodium* parasites.

**FBG binds mosquito midgut peritrophic matrix (PM)**

Previously, we showed that FREP1 bound PM (11). Here we determined which domain (FBG or N-FREP1) is responsible for this interaction. Since PM forms only after a bloodmeal, blood fed-mosquito midguts and naïve mosquito midguts were incubated with BSA, recombinant FREP1, N-FREP1, and FBG separately. Ten midguts were used in each treatment. Three independent experimental replicates were performed. BSA and FREP1 are negative and positive control respectively. Since naïve mosquito midguts do not contain PM, they are used as negative controls as well. After incubation, midguts and their binding proteins were homogenized, and extracts were used to coat an ELISA plate. The bound recombinant FREP1, N-FREP1, and FBG were probed with anti-His monoclonal antibody. After developed with ELISA reagents, FREP1- and FBG- incubated with blood-fed mosquito midguts exhibited significantly ($p$<0.005) higher signal than BSA-incubated midguts. There is no significant ($p$>0.4) difference between N-FREP1 incubated midguts and negative controls (Fig. 6). These results support FBG binding to PM.

**Anti-FBG antibodies prevent FREP1 from binding to *Plasmodium* parasites**

Since anti-FBG anti-serum blocks *Plasmodium* transmission and FBG domain of FREP1 binds *Plasmodium* parasites, anti-FBG anti-serum should inhibit the interaction between FREP1 and parasites. We used ELISA and IFA to test this hypothesis. For ELISA, we coated a plate with the lysate of sexual stage parasites. The purified FREP1 and anti-FBG serum were mixed and added to coated wells. The normal mouse serum replacing anti-FBG anti-serum was used as a negative control (no blocking activity). Incubation of coated plate without FREP1 was used as a positive control (completely blocked). The parasite-bound FREP1 was quantified by antibodies against N-FREP1 protein. Results indicated that anti-FBG anti-serum significantly ($p$<0.01 by t-test) reduced binding signals between FREP1 and parasites (Fig. 7). In order to confirm that anti-FBG anti-serum blocks the interaction between FREP1 and sexual stage parasites, IFA assays were conducted. As shown in Fig. 8, *P. falciparum* gametocytes and ookinetes incubated with FREP1 mixed with anti-FBG serum have much weaker signals than those mixed with pre-immune mouse serum.

## Discussion

Malaria TBVs are novel approaches for malaria control. However, there are few efficacious antigen targets that have been described. We identified a novel *Plasmodium* invasion pathway, e.g. FREP1 mediated *Plasmodium* invasion through direct binding to *P. falciparum* gametocytes and ookinetes (11). Since FREP1 localizes in the mosquito midgut peritrophic matrix, it is readily accessible to antibodies in co-ingested blood. Therefore, mosquito FREP1 is an ideal target for a TBV.

One of the scientific hurdles in developing TBVs is that malaria is caused by multiple species of parasites transmitted by multiple species of mosquito vectors. Identification of an antigen with the potential to limit the transmission of

multiple major parasite species by multiple major vectors is critical for developing a successful TBV. Previously, we demonstrated that anti-FREP1 antibody effectively inhibited the infection of *An. gambiae,* a major malaria vector in Africa, by *P. falciparum,* a major human malaria pathogen (11). Here, we show that anti-FREP1 antibody also significant reduced the transmission of *P. vivax,* another important human malaria pathogen by *An. dirus,* another major mosquito malaria vector in Asia. Moreover, our data show that anti-FREP1 antibodies also inhibit transmission of *P. berghei* to mosquitoes. *P. berghei* is a distantly related parasite causing rodent malaria. Together, we propose that FREP1 is an excellent candidate as a universal TBV antigen.

We examined the orthologs of FREP1 in anopheline mosquitoes. The length of FREP1 orthologs is various, from 311 amino acids in *An. dirus* to 738 amino acids in *An. gambiae*. However, they share a highly conserved FBG domain that is about 210 amino acids in length. Notably, antibodies against FREP1 FBG domain effectively blocked the transmission of *P. berghei* and *P. falciparum* to mosquitoes. Because the *E. coli*-expressed FREP1 FBG domain used for immunization was purified under a denaturing condition and the transmission-blocking activity of anti-FBG antibody was functional, a fully folded FBG is not required as a vaccine antigen. This is an advantage for this highly conserved mosquito midgut antigen (3,13,14). We discovered that FBG domain in FREP1 binds *Plasmodium* gametocytes and ookinetes. Anti-FBG serum prevents parasites from binding to FREP1, which explains the molecular mechanisms of FREP1 FBG as a universal TBV antigen. Contrary to FBG domain, the N-FREP1 did not bind to parasites. Since FREP1 localized in mosquito midgut PM, our data support our prediction (11) that FBG domain interacts with PM as well.

We also investigated the immunogenicity, toxicity and autoimmunity following FREP1 and FBG immunization of mice. Immunization with either *E. coli* or insect cell expressed FREP1 proteins elicited high titer units of antibodies, supporting that recombinant FREP1 and FBG is highly immunogenic. High-titer unit functional antibodies are required for TBVs. The principle mode of action of this approach depends on high levels of antibodies circulating in the blood of the human host at the time that the malaria mosquito vector takes a bite, in order for the antibodies to prevent parasites from invading mosquito guts. Importantly, FBG immunization of mice in a clinically relevant adjuvant (Alhydrogel) did not induce autoimmune reactions, immunopathology, or elicit cross-reactive antibodies against endogenous or human fibrinogens.

Our data collectively demonstrate that FREP1-mediated *Plasmodium* invasion pathway is highly conserved in *Plasmodium* parasites and *Anopheles* mosquitoes. Indeed, a cocktail of vaccine antigens that includes both FBG and the parasite expressed FREP1-binding partners would be predicted to synergistically increase transmission-blocking efficacy and potentially enable the vaccines to completely inhibit malaria transmission.

## Experimental procedures

**Ethics Statement.** This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). Mice were used according to approved protocols (R15-012) by the University of Oklahoma Institutional Animal Care and Use Committee (A3240-01). The *P. vivax* infected patients blood was used to examine the efficacy of antibodies. We followed the NIH Human Subjects Policies and Guidance. The Ethical Review Committee of the Faculty of Tropical Medicine, Mahidol University approved *P. vivax* infection protocols (MUTM 2011-040-05).

**Mosquito and *P. falciparum* maintenance.** *An. gambiae* G3 strain and *An. dirus* were reared in an insectary room maintained at 27°C, 80% humidity with a 12-hr day/night cycle. Larvae were fed with ground fish food (KOI, 0.1mg per larvae per day). Adult mosquitoes were maintained on 8% (w/v) sucrose and fed with mouse blood for egg production. *P. falciparum* parasites (NF54 strain from MR4, Manassas, VA) were maintained in RPMI-1640 medium (Life Tech, Grand Island, NY) supplemented with 10% heat-inactivated (56°C for 45 min) human AB+ serum (Interstate blood bank, Memphis, TN), 12.5 μg/mL hypoxanthine and 4% haematocrit (O+ human blood) in a candle jar at 37°C as described previously (11).

***In vitro* transmission-blocking assay of *P. berghei* infection in *An. gambiae* with anti-FREP1 antibodies.** *P. berghei* (ANKA GFPcon strain) infected mouse blood was precipitated by centrifugation (2000xg for 3 minutes). The blood pellet was then mixed with an equal volume of anti-serum. The standard membrane-feeding assay (SMFA) (15) was conducted using 3-days old female naïve mosquitoes. After feeding for 20 minutes, the engorged mosquitoes were maintained on 8% sugar (w/v) at 19°C. Seven days after infection, mosquitoes were dissected and the midguts were stained with 0.2% mercurochrome and examined using light microscopy to count the number of oocysts. Data were analyzed with nonparametric Wilcoxon test implemented in software R-project.

***In vitro* transmission-blocking assay of *P. vivax* infection in *An. dirus* mosquitoes with anti-FREP1 antibodies.** Field isolates of *P. vivax* were collected from patients attending malaria clinics in Ubonratchanthani province, Thailand. Within 10 hours after collecting blood, aliquots of 350 µl of infected blood were prepared. The infected blood was centrifuged at 1,500 xg for 5 min and plasma was removed. Packed blood was washed once with RPMI-1640 incomplete medium. The antiserum was mixed with *P. vivax* infected packed blood at 1:1 ratio (v/v). The suspension was incubated at room temperature for 15 minutes before being fed to 100 female *An. dirus* (age 5-7 days) per treatment for 30 minutes using membrane feeding device. The packed infected blood mixed with naïve human AB serum at 1:1 ratio (v/v) was used as a control. Engorged mosquitoes were kept on 10% sugar solution. The number of oocysts in mosquito midguts was determined under a microscope at day 7 post bloodmeal.

**Gene cloning, protein expression, and purification**. The full length coding sequence of *An. gambiae* FREP1 was amplified as described previously (11). The FREP1 FBG fragment was amplified with the gene specific primers (5'-ACGCATGCCACGAGTACGAGTCGATC-3', 5'-TGCAAGCTTGTACTCGTGCTCGACCGACTC-3'), PCR fragment was cloned into pQE30 vector and expressed *E. coli* strain M15[pREP4] (Qiagen, Valencia, CA) with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at room temperature. The recombinant protein was purified on a Ni-nitrilotriacetic acid column (Qiagen) and eluted with Buffer D (20 mM Sodium Phosphate, 500 mM NaCl, 8M Urea, PH4.0). The eluted fractions were analyzed on 12% SDS-PAGE gel followed by Coomassie blue staining to examine the purity. All fractions containing protein were then combined and protein concentration was determined using the Bradford protein assay (16).

**Expression of recombinant FREP1 protein in insect cell HighFive cells.** As described previously (11), **t**he full-length FREP1 was cloned into plasmid pIB/V5-His (Life Technologies, Carlsbad, CA). After being amplified in *E. coli* DH5α, plasmids were purified using GenElute Endotoxin-free plasmid preparation kits (Sigma-Aldrich, St. Lois, MO). The purified recombinant plasmids pIB-FREP1 and pIB-chloramphenicol acetyltransferase (CAT) (as a control) were transformed into 40-60% confluent cabbage looper ovarian cell-derived High Five cells. Cellfectin® Reagent (Invitrogen, Carlsbad, CA) was mixed with each individual plasmids (1µl Cellfectin/µg plasmids) in 5-6 ml Express Five® SFM medium (Invitrogen) to transfect into cells. After 5 generations of dilution, the cells that stably expressed the recombinant protein were cultured in 25cm$^2$ CELLSTAR® cell culture flask (Greiner Bio-One, Monroe, NC) for 48 hrs at 27°C. The secreted FREP1 protein in medium was purified using Ni-NTA column and dissolved in PBS for use.

**Immunizations of mice with FREP1 and FBG.** Five Hsd:ND4 female (20-25g; 7 to 8 weeks old) mice were primed via subcutaneous (s.c.) injection and boosted two times at 3-week intervals via (s.c.) injection with 20µg of FREP1 per mouse in 200 µL phosphate buffer saline (PBS) adsorbed (1:1) in Alhydrogel adjuvant. The control mice were primed or boosted with PBS only and adsorbed (1:1) with Alhydrogel. Similarly, five mice were injected (s.c.) with FBG protein under the same regime for optimal-prime boosting with 20µg of the purified recombinant protein in 0.5X buffer D, with control group comprised of five mice injected with 0.5X buffer D adsorbed in alhydrogel instead. Serum was collected from each mouse prior to each priming and boosting immunization. Animals were sacrificed following *in vivo* feeding assays, and blood was collected via orbital sinus (17). Blood samples were centrifuged

at 2,000x g, for 20 minutes at 4ºC for serum separation.

***In vivo* transmission-blocking assay with the immunized mice.** Mice were immunized with the same regime for optimal-prime boosting as described above. Ten days after second boost, naïve and optimally prime-boosted Hsd:ND4 mice were infected via i.p. route with *P. berghei*–infected blood. When parasitemia reached <6% mice were injected with phenylhydrazine hydrochloride (60 mg/Kg) (Santa Cruz Biotechnology, Dallas, Texas) to stimulate gametocytemia. Mice harboring circulating gametocytes (confirmed by Giemsa staining) were anesthetized and used to feed 100 3-day old *An. gambiae* female mosquitoes for 20 minutes. After feeding, un-engorged mosquitoes were removed and engorged mosquitoes transferred to a cage and kept at 19$^{o}$C and 8% (w/v) sucrose. Seven days after infection, mosquitoes were dissected and the midguts were stained with 0.2% mercurochrome and examined using light microscopy to count the number of oocysts. Two independent experiments were performed for each treatment.

**Determining antibody titer units with ELISA assays.** Mice sera were collected and assayed for the presence of FREP1 specific IgG antibodies using ELISA. In brief, recombinant affinity-purified FREP1 was diluted to 0.5µg/ml in 0.1M sodium phosphate ($Na_2HPO_4$), pH 9.0 binding buffer and used to coat 96-well plate MediSorp$^{TM}$ plates (NUNC –Denmark) overnight at 4 $^{o}$C. Wells were washed three times with 0.2% Tween 20 in PBS (PBST) and blocked with 2.5% bovine serum albumin (BSA) and 1% normal goat serum in PBS for 2 hrs at room temperature. Serum samples were prepared by 1:5 serial dilutions starting at 1:100 to 1:8x10$^{6}$, 50 µl were added into each wells and incubated at room temperature for 1 hr. Bound IgG antibodies were detected using AP-conjugated goat anti-mouse IgG (Sigma-Aldrich) at 1:800 in blocking solution for 2 hrs at room temperature and visualized using p-Nitrophenylphosphate (Sigma-Aldrich, St. Louis, MO) as the substrate. Absorbance at 450nm was measured using Epoch microplate spectrophotometer (Biotek, Winooski, VT). Antibody titer unit was expressed as endpoint titer unit, the highest dilution of serum that gives a reading above the cutoff (2x standard deviation of the signal generated from pre-immune serum).

**Cross-reactivity of mouse FREP1 serum to human plasma fibrinogen.** Human blood (AB+) was purchased from Oklahoma Blood Institute. Whole blood collected from donors were transferred into BD Vacutainer® blood collection tubes (Becton and Dickinson Company, Franklin Lakes, NJ) coated with ethylenediaminetetraacidic acid dipotassium salt dihydrate ($K_2$EDTA) to prevent coagulation. Blood sample was centrifuged at 3,000x g for 5 minutes at 4ºC to separate plasma. 50µL of human plasma samples without dilution (1) or diluted 10-fold in PBS (2) were coated overnight at 4 ºC in a 96-well plate (Brand, Wertheim, Germany). 50µL insect cell-expressed recombinant FREP1 (0.5µg/ml) and BSA (10mg/ml) were coated as positive and negative controls respectively. Samples were diluted in 0.1M $Na_2HPO_4$ (pH 9.0). Each well was then incubated with the following solutions at RT: 150 µl blocking buffer (2.5% BSA and 1% normal goat serum in PBS) for 2 hrs, 50 µl of anti-FREP1 mouse serum (1:1,000), control mouse serum (1:1,000) and anti-FREP1 rabbit serum (1:2,000) diluted in blocking buffer for 1 hr, 50µL of alkaline phosphatase-conjugated goat anti-mouse IgG (1:800) and goat anti-rabbit IgG (1:20,000) for 1.5 hrs. Wells were washed for 5 minutes with PBST three times between incubations. At the end, the samples were developed with 50 µl of p-Nitrophenylphosphate solution (Sigma-Aldrich). Three replicates were each sample.

**Immunofluorescence assays (IFA).** Immunofluorescence assays were performed as described previously (11). In brief, *P. falciparum* cultures, containing cells and parasites, were smeared on coverslips (Fisher Scientific, Waltham, MA) and fixed in 4% paraformaldehyde in PBS at room temperature for 30 minutes. After quenching with 0.1M glycine in PBS, coverslips were blocked overnight at 4ºC in 2.5 % BSA and 1% normal goat serum in PBS (blocking solution). Cells were incubated for 2 hours at room temperature with High Five cell-expressed FBG (100µg/ml) in blocking solution. Sequentially, cells were incubated for 1 hour with mouse monoclonal anti-His antibody (1:1, 000) and Goat anti-mouse (1:1, 000) secondary antibody (Alexa Fluor® 555, Life Technologies, Inc., Carlsbad, CA) for 45 mins, both diluted in blocking solution. Cells were washed between each step three times for 5 mins in PBST. Cells in the control group

were incubated with High Five expressed 100 µg/ml chloramphemicol acetyltransferase (CAT). Coverslips were mounted on glass slides using 20µL vectashield mounting media (Vector Laboratories, Burlingame, CA) and visualized using a Nikon Eclipse Ti fluorescence microscope.

**Alignment of FREP1 sequences from multiple species of anopheline mosquitoes.** The orthologs of FREP1 in various anopheline species were obtained from the Vector-Base genome server (18). The multiple sequence alignment of *An. gambiae* FREP1 with its orthologs was built using ClustalO program ver. 1.2.1(19) and visualized with Jalview (20).

**Using ELISA approach to demonstrate that anti-FBG serum inhibits the interaction between FREP1 and parasites.** 15-day cultured *P. falciparum* NF54 cells were lysed in PBST. About 50µL of supernatant (2.0mg/ml) per well was used to coat a 96-well plate. Purified recombinant full-length FREP1 was mixed with pre-immune mouse serum (1:150) or anti-FBG serum (1:150, final titer units: $1x10^4$). BSA (2.5 mg/mL) was used as a negative control. The bound FREP1 in wells was probed with anti-N-FREP1 mouse serum (1:1000 dilution), followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG (1:20,000). After developed with pNPP (Sigma), $OD_{405}$ was measured using Epoch microplate reader. Three replicates were conducted.

**Binding assays between FBG and PM**. 3-5-day old G3 *An. gambiae* mosquitoes were fed with mouse blood. The engorged mosquitoes were maintained in insectary ($27^0$C, 80% humidity) for 18 hrs. Ice-anesthetized mosquitoes were then dissected to obtain midguts, and the blood bolus inside a midgut was removed by puncturing it. Blood fed-mosquito midguts and naïve mosquito midguts were incubated with 200µL of 0.1mg/ml BSA, full-length FREP1, N-FREP1, and FBG separately for 2 hrs at RT. Ten midguts were used in each treatment. Three independent experimental replicates were performed. After incubation, midguts were homogenized with pestles in 300µL of 0.5% Tween-20 in PBS. Midgut lysate supernatants (50µL) were used to coat 96-well plates overnight at 4ºC. Since recombinant proteins of FREP1, FBG, N-FREP1 contain His-tag, the bound protein was probed with 50µL of mouse anti-His monoclonal antibody and developed as we mentioned above.

**Statistical Analyses**: As described previously (21), the *p* values of oocyst difference for in vivo infection assays and SMFA were obtained with nonparametric Mann-Whitney-Wilcoxon test using R package software. The p value for binding assays were obtained using two-tailed Student's t-test. The *p* values of cross reactions triggered by FREP1 were calculated with one-way ANOVA in R package software.


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## Conflict of Interest

The authors have declared that no competing interests exist.

## Author contribution

GN and CF prepared proteins, vaccinated mice and infected mice and mosquitoes. GZ involved in infecting *An. gambiae* mosquitoes. WR and WN and JP designed and conducted experiments related to *P. vivax* and *An. dirus*. XW participated data analysis. NSB involved in experimental design and vaccinating mice. JL designed the project, conducted experiments, and analyzed the data. GN, CF and JL wrote the manuscripts. All edited the manuscript.

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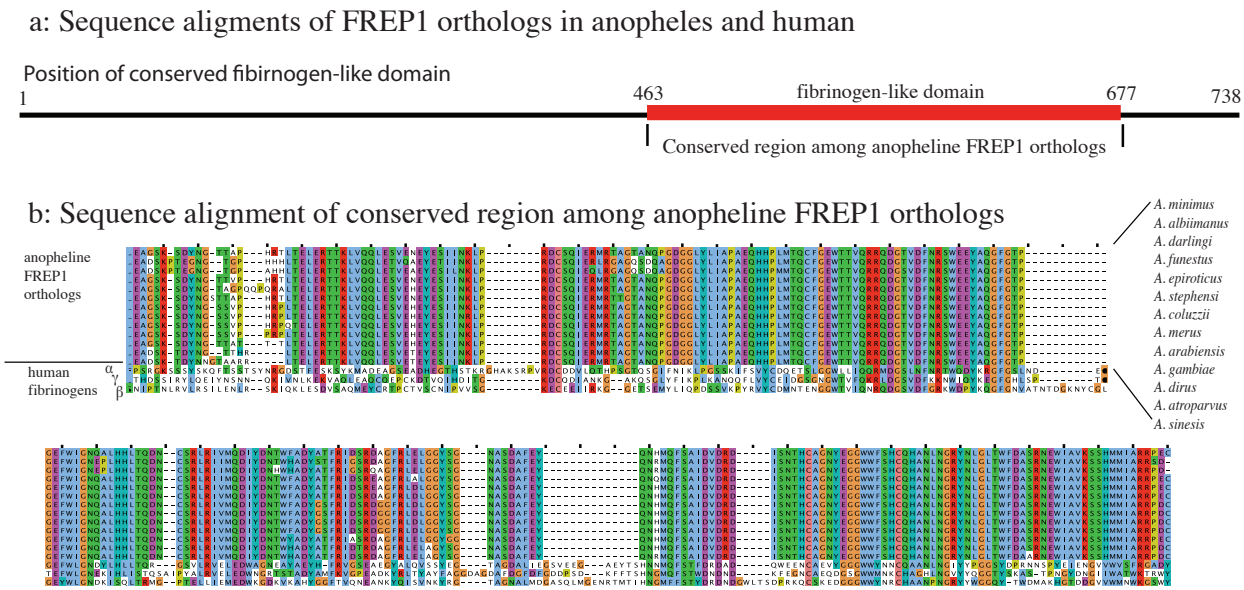


**Fig. 1: Multiple sequence alignment of FREP1 from *An. gambiae* and other major malaria vectors**. **a)**: The overview of sequence alignments of FREP1 orthologs in *Anopheles* mosquitoes and human fibrinogens. **b)**: Detailed alignment of conserved FBG domains. Dots and dashes are insertions or deletions. Clustal X colour scheme is used to depict letters if the amino acid profile of the alignment at that position meets criteria and residue types, which highlights the conservations.

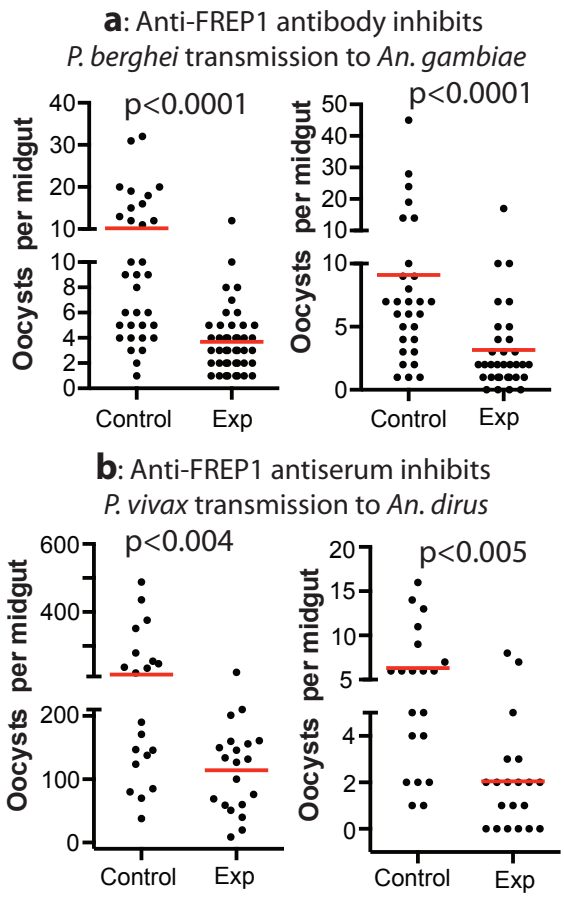


**Fig. 2: Rabbit anti-FREP1 anti-serum inhibits *P. berghei* (a) and *P. vivax* (b) infection of *An. gambiae* and *An. dirus mosquitoes*, respectively. Control:** Pre-immune rabbit serum was used. **Experiment (Exp)**: anti-FREP1 rabbit serum was used. The *P* values were calculated using Mann-Whitney-Wilcoxon test.

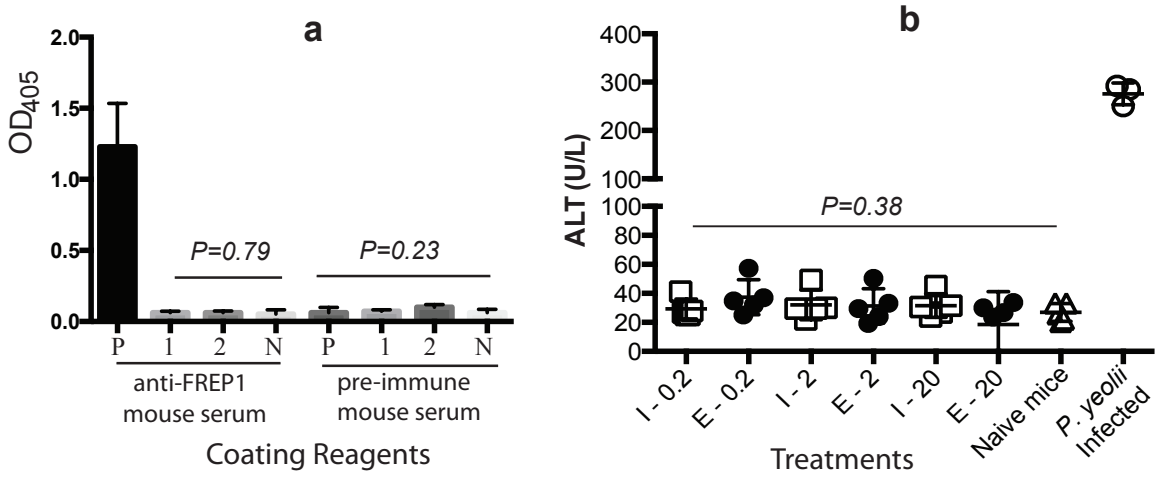


**Fig. 3: FREP1 immunizations do not trigger autoimmune reactions against mammalian or human fibrinogens and are nontoxic to mice**. **a):** Mouse anti-FREP1 antiserum does not cross-react with human fibrinogens, and no autoimmunity was induced either. P: coated with insect cell-expressed recombinant FREP1; 1: coated with human plasma; 2: coated with 10-fold diluted human plasma; N: wells coated with BSA. **b):** The serum ALT activity does not change significantly between the FREP1-immunized mice and control mice. I-: insect cell expressed recombinant FREP1. E-: *E. coli* expressed recombinant FREP1. The *p* values were calculated with one-way ANOVA.

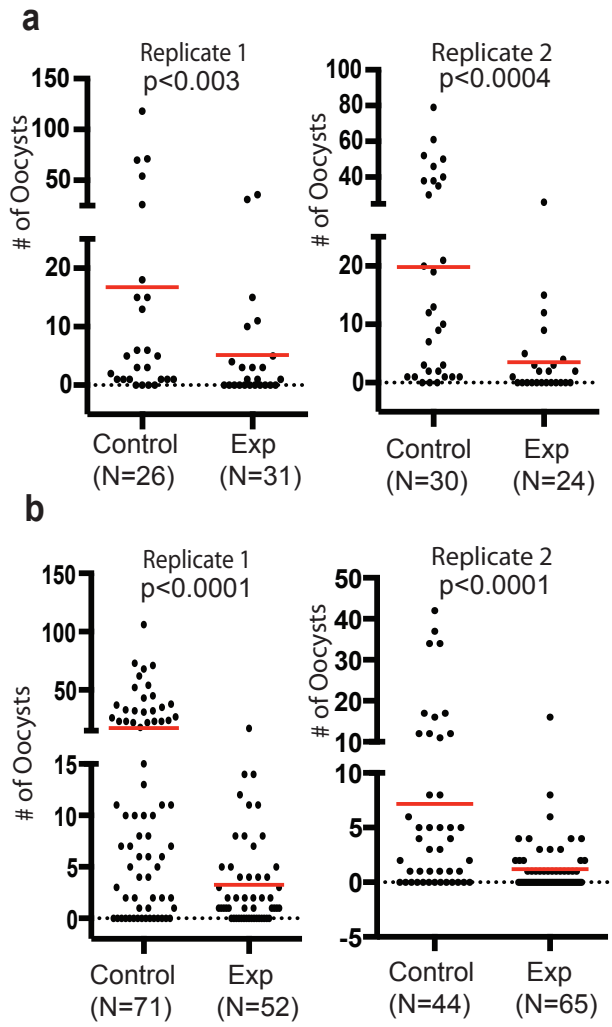


**Fig. 4: Immunization of mice with purified FBG formulated with Alhydrogel inhibits *P. berghei* transmission to *A. gambiae* in *in vivo* studies (a) and *P. falciparum* transmission to *A. gambiae in vitro* with SMFA (b). a):** Results of direct feeding assays using two FBG-immune mice and two control mice showed the transmission-blocking activity of *in vivo* generated anti-FBG antibody response against *P. berghei* to *An. gambiae* mosquitoes. **b):** Serum collected from FBG-immune mice also blocks *P. falciparum* (NF54 strain) infection to *An gambiae* mosquitoes. Control or anti-FREP1 immunized mouse sera were mixed with human serum (1:4 ratio) and mixed with *P. falciparum*-infected packed human blood prior to its use in SMFA. The final anti-FBG antibody titer was $4x10^5$. The *P* values were calculated using Mann-Whitney-Wilcoxon test.

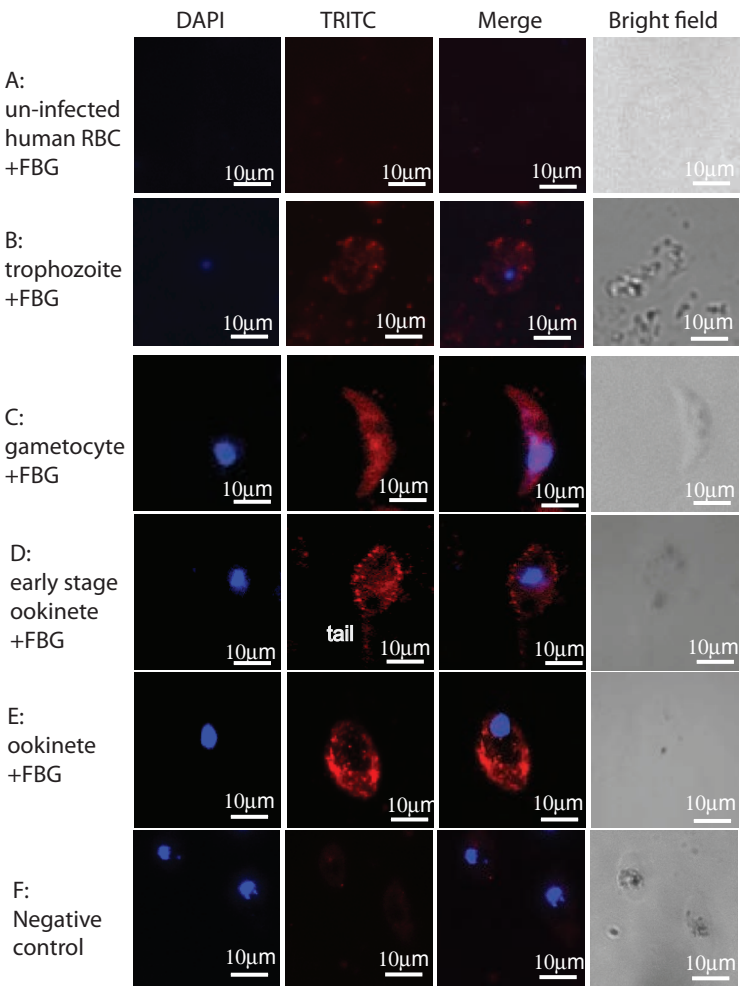


**Fig. 5: Insect cell-expressed recombinant FREP1 FBG protein interacts with *Plasmodium falciparum* detected by indirect immunofluorescence assays (IFA).**
The first and second columns depict cell nuclei stained with DAPI and binding FBG proteins, respectively. Merging column one and two generated the third column, showing the co-localization of *P. falciparum* (nuclei) and FBG protein binding. The 4th column shows the bright views of the cells. Row A: FBG does not bind to un-infected RBC. Row B: the interaction between FBG and asexual *P. falciparum* is weak. Row C: FBG binds to *P. falciparum* gametocytes. Row D, E: FBG binds to *P. falciparum* ookinetes. Row F: An irrelevant control protein (CAT) does not bind *P. falciparum* parasites.

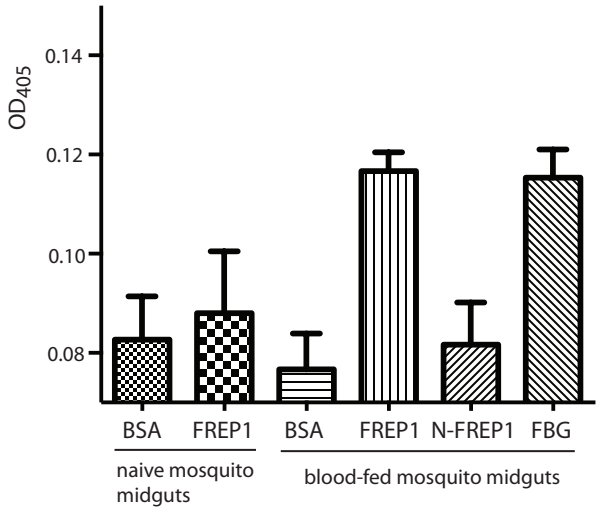


**Fig. 6: FBG binds to mosquito peritrophic membrane (PM).** Mosquito midguts were obtained from naïve mosquitoes that do not have PM (as a negative control) and from 18-hour post blood-fed mosquitoes that contain PM. BSA (negative control) and recombinant proteins of FREP1 (positive control), N-FREP1, and FBG were incubated with midguts. After removing the supernatant, the pellets extracted and the remaining recombinant proteins were developed with anti-His monoclonal antibodies.

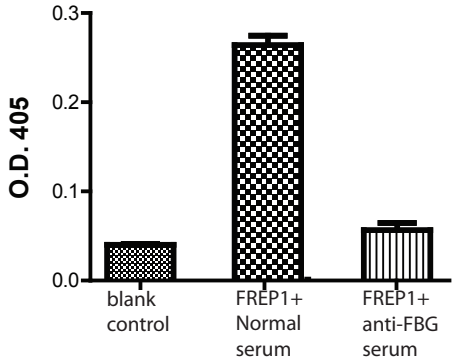


**Fig. 7: Anti-FBG antibodies blocked the interaction between mosquito peritrophic membrane and recombinant FREP1 protein.** Eighteen-hour post bloodmeal mosquito midguts were incubated separately with BSA (blank control), recombinant FREP1 pre-mixed with pre-immune mouse serum, and recombinant FREP1 pre-mixed with anti-FBG mouse serum. After removing the supernatant, the midgut-bound recombinant FREP1 was quantified by antibodies against N-FREP1 protein using ELISA.

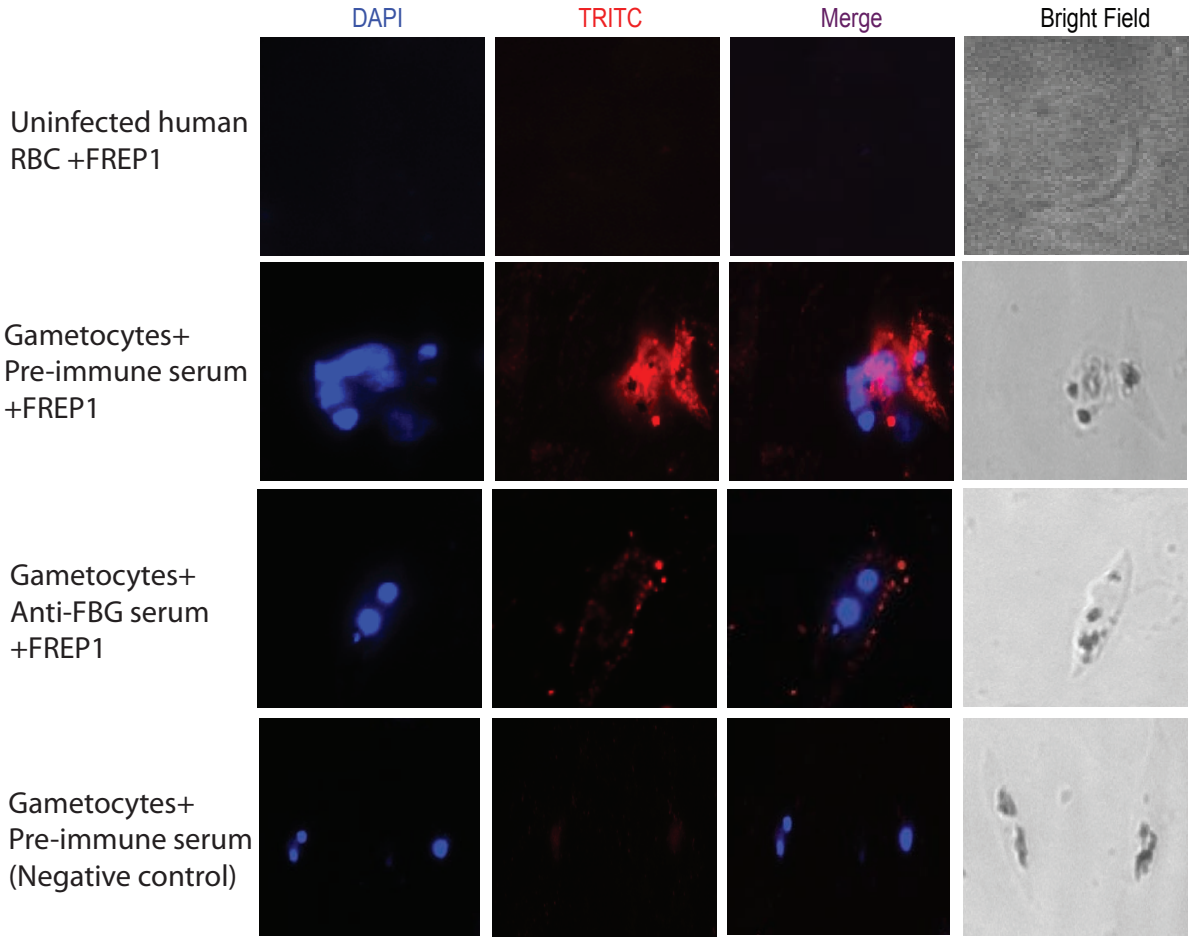


**Fig. 8: Anti-FBG antibody prevents FREP1 from binding to parasites.** The stage V gametocytes were deposited onto glass coverslips, and incubated with FREP1 mixed with pre-immune mouse serum (2nd row) or anti-FBG mouse serum (3rd row). The bound recombinant FREP1 protein was quantified by anti-His monoclonal antibody. 1st row shows uninfected human red blood cells do not bind FREP1. The same experiments as 2nd row without FREP1 showed no red signals (4th row).

# Fibrinogen domain of FREP1 is a broad spectrum malaria transmission-blocking vaccine antigen

Guodong Niu, Caio Franca, Genwei Zhang, Wanlapa Roobsoong, Wang Nguitragool, Xiaohong Wang, Jetsumon Prachumsri, Noah S. Butler and Jun Li

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